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Y Chromosome Regulation of Autism Susceptibility Genes

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14. ABSTRACT Autism spectrum disorders (ASD) are a group of pervasive neurodevelopmental disorders with impairments in social interaction, language and range of interests. There is a significant sexual dimorphism with affected boy to girl ratios as high as 8:1. Recent studies in our laboratory demonstrated that the Y-located transcription factor, SRY, could possibly play a genetic modifier role in the expression of several significant autism susceptibility genes (ASGs). The major goal of the project is to confirm that SRY indeed can possibly influence the expression of these autism susceptibility genes, by demonstrating its binding to the promoters, influencing the expression of ASGs, and exploring additional potential neuronal targets for SRY in neuronal cell cultures. Various molecular techniques will be used for these studies, whose results should provide critical insights on the roles of SRY and the Y chromosome genes in dysregulation of ASGs, and their contributions to the etiologies of ASD, thereby explaining the sexual dimorphism in male susceptibility to the disease.					
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INTROUDCTION

Autistic spectrum disorders (ASD) are a group of pervasive neurodevelopmental disorders with impairments in social interaction, language and range of interests, and a multitude of co-morbidities, including gastrointestinal ailments, immune dysfunction, motor and sensory deficits and metabolic dysfunctions [1-7]. The etiologies of ASD are currently unknown. Genetic linkage analyses and whole genome association studies (WGAS) have identified genomic copy number variation (CNV) and numerous autism susceptibility loci and genes, suggesting a multi-genetic nature of ASD [2, 8-11]. These autism susceptibility genes/loci could play important roles in the development and physiology of the central, sympathetic, enteric and sensory nervous systems. Mutations within the autism susceptibility genes contribute to autistic development, particularly the severe or syndromic form of autism whose phenotypes could be recognized readily by trained clinicians. A large portion of autistic children, however, possesses the high-functioning form of autism with symptoms that require specific and standardized diagnostic testing [1]. The etiologies of high-functioning autism are unclear. Epigenetic dysregulation of the same autism susceptibility loci/genes and/or additive effects of genetic and epigenetic changes could contribute to the development of this form(s) of autism. Significantly, there is a distorted ratio of boys to girls among high-functioning autistic children. Such distorted ratio could be as high as 8:1 in some populations [12, 13]. Sexual dimorphism in autism is a key and consistent feature among affected children. It parallels numerous observations of sex differences in both neural anatomy and psychiatry between males and females [14-17]. Currently, the exact mechanisms leading to such sexual dimorphisms in ASD are unknown. Both sex hormone influences and differential actions of genes on the sex chromosomes have been postulated to play key roles in sexual dimorphisms in normal and neural/cognitive disorders, including autism [18-24]. Recent studies in our laboratory suggest that genes on the Y chromosome could contribute to such sexual dimorphisms, thereby raising the possibility of a genetic and/or epigenetic basis for sexual dimorphism in both normal and diseased physiology.

Among the Y chromosome genes, the sex-determining region Y (SRY) gene [25] is the most significant candidate capable of exerting sexual dimorphisms in humans. SRY is responsible for switching on male sex differentiation, and is absolutely required for differentiating the fetal gonads into testes [26, 27], but perhaps not other tissues. It encodes a dual-function transcription factor capable of simultaneously repressing the ovarian and activating the testis determining genes in the fetal gonads. It harbors a DNA binding HMG box of ~80 amino acids and is the founder of a family of fate-determining genes, SRY-related HMG box (SOX) genes. The HMG boxes among SRY/SOX genes are functionally interchangeable [28], suggesting that the flanking sequences/domains and the regulation of their spatiotemporal expression could play key roles in the respective biological functions. Significantly, SRY does not contain any transcription domain while most SOX proteins do [29]. SRY recruits either co-repressors or co-activators and forms transcription complexes capable of mediating gene repression and activation respectively [30-32]. Most SOX proteins could regulate the respective target genes either independently or in complexes with other co-factors. Since SOX genes, particularly SOX-E genes (SOX8, 9, and 10), serve important roles in neurogenesis and brain physiology [33-36], ectopic expression of SRY in neural tissues could compete against the SOX gene actions and could have significant consequences, depending on the co-regulators it might recruit. As evidenced by our preliminary results, SRY binds to the promoters of numerous neural genes, including many of the autism susceptibility genes, and dysregulates their expression,

thereby positioning SRY as a key male-specific genetic modifier/mediator on the Y chromosome for susceptibility to ASD development.

To identify the targets for the SRY transcriptional complexes, we have conducted a series of chromatin immunoprecipitation and genome-wide promoter tiling microarray (ChIP-Chip) experiments with gonadal cells isolated from mouse embryos at the time of sex determination [37, 38]. We identified ~2000 targets (a normal number for most global ChIP-Chip studies), which include numerous known ovarian and testicular differentiating genes. We have selected ~120 targets for further gene-specific analysis and confirmed SRY binding to the promoters of ~88% of these genes. Significantly, gene ontology analysis [39] identified 164 genes that play certain functions in nervous system development, neurogenesis, axonogenesis, axon guidance, synaptic transmission and neurotransmitter receptor activities. Importantly, numerous autism susceptibility genes, including the monoamine oxidase A (MOAA), mediator complex subunit 12 (MED12), homeobox B1 (HOXB1) gastrin-releasing peptide receptor (GRPR), γ -aminobutyric acid (GABA) A receptor b3 (GABRB3), vasoactive intestinal peptide receptor 2 (VIPR2), autism susceptibility 2 (AUTS2), disrupted in schizophrenia 1 (DISC1), neuroligin 1 and 2, glial cell derived neurotrophic factor (GDNF) and its RET tyrosine kinase receptor, were among the SRY neural targets. The bindings of the SRY transcription complexes to these neural genes suggest that they could be involved in either ovarian or testicular differentiation [40, 41]. Alternatively, gonadal progenitors could be multipotent and possess certain neural lineage, as supported by findings that certain testicular embryonal teratocarcinoma cell lines, such as NT2/D1, are capable of differentiating into neurons or glial cells upon appropriate inductions [42, 43]. We hypothesize that low/moderate levels of SRY expression could result in sexual dimorphisms in physiology and neuroanatomy, while ectopic/aberrant levels of SRY expression could predispose boys at a higher frequency than girls to the development of autism and other cognitive disorders.

BODY

Task 1. To confirm the direct SRY binding to the promoters of respective autism susceptibility genes.

SRY binding to promoters of autism susceptibility genes

To confirm the Sry binding to autism susceptibility genes, DNAs were extracted from SRY-antibody immunoprecipitated chromatin, mock-immunoprecipitated chromatin with a normal antibody, and input chromatin, and were analyzed with specific primers derived from the promoter sequences of the respective autism susceptibility genes, including AUST2, HOXB1, GDNF, VIPR2, MAOA, MED12, RET and GRPR. Our results showed that promoter-specific DNA fragments were amplified with the input and SRY-ChIP DNAs, but not those with the mock-ChIP DNA nor water (Table 1), suggesting that SRY indeed binds to the promoters of these autism susceptibility genes, thereby raising the possibility that this Y-encoded transcription factor could indeed regulate the expression of these autism susceptibility genes.

Table 1. ChIP-Chip Confirmation by PCR & Gene-Specific Primers

	Input	Mock	ChIP	Water
AUTS2				
HOXB1				
GDNF				
VIPR2				
MAOA				
MED12				
RET				
GRPR				

Task 2. To evaluate the effects of SRY on expression of autism susceptibility genes in neuronal culture.

Confirmation of SRY binding to the promoters of above autism susceptibility genes suggests that SRY could indeed regulate their expression. To demonstrate such potential effects of SRY, we have initially selected two autism susceptibility genes, the RET proto-oncogene and monoamine oxidase A (MAOA) gene for detail studies. MAOA deaminates monoamines and is involved in the metabolism of neurotransmitters, such as serotonin. Mutations and polymorphism of MAOA gene have been associated with various forms of cognitive disorders, including autism, attention deficit and hyperactivity disorders, and schizophrenia. The RET proto-oncogene serves important functions in enteric, central and peripheral nervous developments. Significant loss of function mutations of RET gene are responsible for both familial and sporadic forms of Hirschsprung's disease, or aganglionic megacolon disease. In most cases, haploinsufficiency of RET product has been postulated to be responsible for under or absence of enteric nervous system development. Since gastrointestinal ailments are frequent features of numerous children affected by high-functioning autism, SRY impairment of RET expression could play a key role in developments of gastrointestinal ailments in autism. Based on these considerations, we have selected these two autism susceptibility genes for characterization.

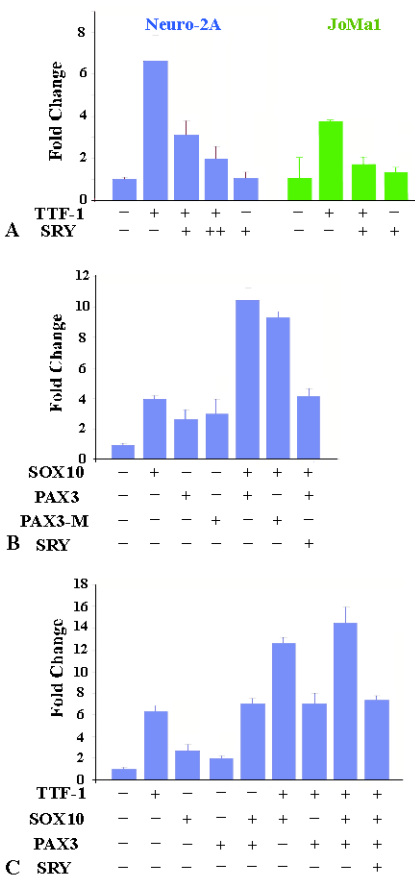


Figure 1. SRY effects on TTF-1 and SOX10-PAX3 stimulations of RET promoter-directed reporter expression. A) SRY suppresses TTF-1 transactivation at the of proximal RET promoter in Neuro-2a neuroblastoma (blue) and JoMa1 neural crest stem cells (green). B) SRY suppresses SOX10-PAX3 stimulation of distal RET promoter. C) TTF-1 and SOX10-PAX3 synergistic stimulation of RET promoter is suppressed by SRY.

a) SRY represses the RET gene activation by TTF-1, SOX10 and PAX3 transcription factors

GDNF family ligands signal through the canonical tyrosine kinase receptor, RET, and co-receptors, GFRa, and play critical roles in neural crest cell migration and ganglion development in the enteric nervous system (ENS); and axonal growth, guidance, survival and synapse formation in sensory and sympathetic, hippocampal, cortical GABAergic and spinal chord neurogenesis [44-46]. SRY binding to their promoters suggests that SRY could modulate their expression in neurogenic tissues. Various studies demonstrated that the RET promoter is regulated principally by a distal and a proximal enhancer domain with two resident transcription factors, i.e. paired box 3 (PAX3) [47, 48] and thyroid transcription factor 1 (TTF-1) [49] respectively. SOX10, a key regulator for ENS development, interacts with both PAX3 and TTF-1 and synergistically transactivates the RET promoter.

SRY represses TTF-1 and SOX10-PAX3 transactivation of a reporter gene directed by the RET promoter

Using a 3.4-kb RET promoter-directed luciferase reporter gene and the human SRY expression construct, we have analyzed the effects of SRY on RET promoter activities in the neuronal cell line, Neuro-2A and a neural crest stem cell line, JoMa1. These expression constructs were transiently transfected to respective cells in various combinations together with an internal control Renilla luciferase construct. The RET-promoter directed firefly luciferase activities were then normalized with the internal control. Our results showed that RET promoter is activated by TTF-1, and SOX10-PAX3 transcription complexes, and such transactivation is repressed by the presence of SRY in a dosage dependent manner (Figure 1).

SRY interacts PAX3 via its HMG box

To explore the possible mechanisms involved SRY repression of both the TTF-1 and SOX10-PAX3 transactivation of RET promoter, we have conducted various co-transfections and protein binding assays to determine if SRY can physically compete with transcription complex formations. SOX10 interacts with PAX3 via its HMG box, forms a transcription complex, responsible for stimulating RET promoter activities. The HMG boxes of SOX10 and SRY are highly homologous, but SRY lacks an acidic domain as that at the carboxyl terminus of SOX10, and is incapable of any transactivation. We postulate that SRY could competitively displace SOX10 from an active PAX3-SOX10 complex, and recruit a KRAB-O-KAP1-HP1 repressor complex, thereby suppressing PAX3-SOX10 transactivation of RET. To support this postulation, we seek to demonstrate that SRY indeed interacts with PAX3 via its HMG box.

To demonstrate an in vivo interaction between SRY and PAX3, we transiently transfected Neuro2A cells with epitope-tagged expression vectors for human FLAG-SRY and Myc-PAX3, and performed immunofluorescence and co-immunoprecipitation and western blot studies with the transfected cells. Our results showed that SRY and PAX3 were co-localized in the nuclei of transfected cells (Figure 2, A-D). Total lysates were prepared from the same cells and immunoprecipitated with specific antibody against FLAG-SRY. The immunoprecipitated proteins were then analyzed by western blotting with antibodies against Myc-PAX3 and FLAG-SRY respectively. Our results showed that PAX3 was co-immunoprecipitated with SRY (Figure 2, I), suggesting that these two proteins interacted in the transfected cells. To determine the domain(s) responsible for interacting with PAX3, GST-fusion proteins containing the human SRY (hSRY), mouse (mSry), HMG box, the bridge (flanking) domain, were used in pulldown assays with ³⁵S-labeled PAX3 protein, synthesized by in vitro transcription and translation reactions. Our results showed that the SRY interacts with PAX3 at its HMG box (Figure 2, K).

SRY interacts with TTF-1 via its HMG box

Using a reporter assay system, we demonstrated that SRY could repress the TTF-1 dependent transactivation of the RET promoter (Figure 1). We surmise that SRY can bind directly to TTF-1, thereby having a more direct influence on TTF-1 transactivation of the RET gene. To explore this possibility, we had conducted similar experiments as those in PAX3-SRY analysis to demonstrate a direct interaction between SRY and TTF-1.

Transiently transfected epitope-tagged human FLAG-SRY and TTF-1-V5 proteins were co-localized in the nuclei of the host cells (Figure 2, E-H). TTF-1 was co-immunoprecipitated with an antibody against the human SRY, as demonstrated with western blotting of precipitated proteins from lysates of transfected cells (Figure 2, J). Again, GST pulldown assays showed that the HMG box of the SRY protein was responsible for such interaction between SRY and TTF-1 (Figure 2, L). Hence, SRY interacts directly with TTF-1 via its HMG box and affects TTF-1 transactivation of RET.

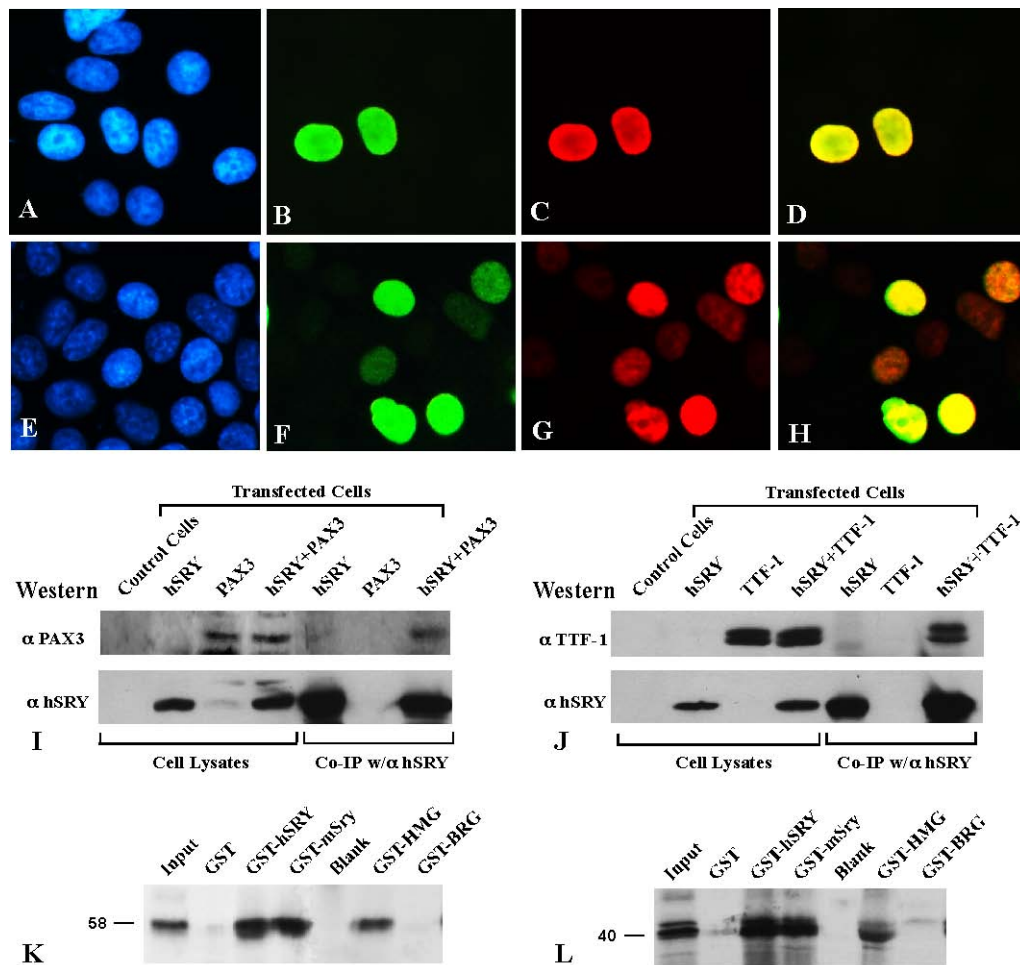


Figure 2. SRY interactions with PAX3 and TTF-1 in vivo and in vitro. SRY co-localizes with PAX3 (A-D) and TTF-1 (E-H) in transfected Neuro2A cells. SRY green, PAX3 and TTF-1 red, D and H are respective merged images. Co-IP of I) PAX3 and J) TTF-1 with hSRY in cells transfected with respective expression vectors. GST pulldowns of K) PAX3 and L) TTF-1 with GST-SRY proteins, showing the HMG box as the interactive domain for SRY.

Loss of function mutations and/or haploinsufficiency of RET are responsible for 50% of familial and 7-35% of sporadic cases of Hirschsprung's disease (aganglionic megacolon), a condition caused by the absence of ganglions in certain segment of the colon [50-52]. Hence, SRY dysregulation of RET could partially contribute to the disease process. Importantly, there is a distorted ratio of 4:1 in boys to girls in Hirschsprung's disease and gastrointestinal ailments are closely associated with autism. Hence, SRY repressions of RET in the enteric, sensory and sympathetic nervous systems could indeed contribute to the complex etiologies of ASD.

b) SRY exacerbates the Sp1 transactivation of monoamine oxidase A (MAOA) gene

The X-encoded MAOA catalyzes the oxidative deamination of monoamine neurotransmitters, such as serotonin, and is critically important in brain development and functions [53]. MAOA is mainly regulated by the interplays of the Sp1 family of transcription factors binding to several Sp1 elements at its core promoter within 240-bp upstream of transcription start site [54]. We have collaborated with Dr. Jean Shih, a key investigator in MAOA field, and examined the effects of SRY on MAOA expression in a neuroblastoma cell line, BE(2)C, using reporter assays, siRNA and chromatin

immunoprecipitation techniques. Our study demonstrated that SRY stimulates MAOA expression at both RNA and enzyme levels and up-regulates MAOA promoter activities (Figure 3A-B). It interacts with Sp1 and synergistically up-regulates MAOA promoter activities (Figure 3C-D). Since variations of MAOA activities have been linked to depression, aggression, schizophrenia, autism and attention deficit and hyperactivity disorder [55-61], SRY could be a genetic modifier in these MAOA associated cognitive disorders, thereby exerting a sexually dimorphic effect(s).

FUTURE DIRECTIONS

The gender difference in high-functioning autism has long been a key phenomenon of the disease. Yet, the exact mechanisms for such sexual dimorphism are uncertain. Various hypotheses have been proposed, including X dosage differences and hormonal influences. The role of the Y chromosome, and the genes therein, has not been explored. Our preliminary studies demonstrated that SRY could possibly bind to genes, postulated to contribute to autism susceptibility, have provided a critical clue that the male-only chromosome is potentially a key player in the sexual dimorphism in high-functioning autism.

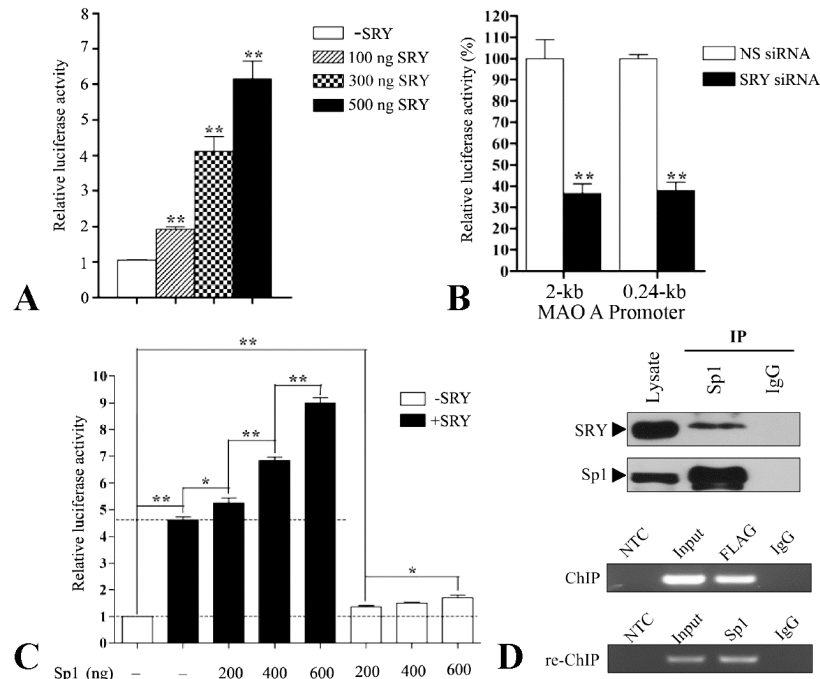


Figure 3. A) SRY stimulates MAOA promoter-directed luciferase expression in BE(2)C cells. B) siRNA knockdown of endogenous SRY represses reporter expression directed by a 2-kb and 0.24-kb MAOA promoter. C) SRY interacts with Sp1 (not shown) and synergistically transactivates MAOA promoter. D) ChIP and re-ChIP analysis showing SRY-Sp1 binding to MAOA promoter as a transcriptional complex on 0.24-kb MAOA promoter.

Since the funding of this Concept project, we have established evidence, supporting SRY binding to the promoters of the autism susceptibility genes, and showed that SRY bindings to 2 of these genes result in dysregulation of the target genes. For the remainder of the 6 months, under no-cost extension for this one-year project, we seek to identify additional neuronal targets for SRY bind performing an additional ChIP-Chip experiment in human neuronal culture cells, thereby providing additional neuronal genes whose expression and presumably functions could be affected by ectopic SRY expression in neural tissues.

KEY RESEARCH ACCOMPLISHMENTS

- Confirm binding of the Y-chromosome encoded SRY transcription factor binding to autism susceptibility genes.
- Demonstrate SRY dysregulation of RET gene in a reporter system in neuronal cells.
- Demonstrate SRY dysregulation of MAOA gene in neuronal cells.
- Provide preliminary evidence supporting the hypothesis that ectopic SRY expression could dysregulate autism susceptibility genes, thereby contributing to their dysregulation during neurogenesis and autism development.
- Provide a plausible explanation for sexual dimorphism in high-functioning autism.

REPORTABLE OUTCOMES

Manuscripts are being submitted or in preparation.

CONCLUSION

Our initial genome-wide studies demonstrating the SRY binding to the promoters of selected autism susceptibility genes have been confirmed by the current project. Further, we showed that SRY binding to 2 of these autism susceptibility genes results in their dysregulation in neuronal cells. These data support our hypothesis that the Y-encoded SRY transcription factor, when ectopically expressed in neural tissues, during antenatal or postnatal development, might dysregulate autism susceptibility genes important for neural development and/or physiology, thereby contribute to the complex etiology of autism, especially the high-functioning autism, which has a significant sexual dimorphism favoring boys.

SO WHAT

Gender difference is a key phenomenon in high-functioning autism. Currently, mechanism(s) for such sexual dimorphism is unknown. Results from the present study demonstrate that the Y-encoded transcription factor, SRY, is capable of binding to autism susceptibility genes, and dysregulate their expression in neuronal cells, thereby supporting the hypothesis that the Y chromosome, and genes therein, could play key roles in autism development. The current project lays a foundation for future studies on Y chromosome genes in neuronal development, autism etiology and sexual dimorphism.

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